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# GATA-2 Plays Two Functionally Distinct Roles during the Ontogeny of Hematopoietic Stem Cells

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## Abstract

GATA-2 is an essential transcription factor in the hematopoietic system that is expressed in hematopoietic stem cells (HSCs) and progenitors. Complete deficiency of GATA-2 in the mouse leads to severe anemia and embryonic lethality. The role of GATA-2 and dosage effects of this transcription factor in HSC development within the embryo and adult are largely unexplored. Here we examined the effects of *GATA-2* gene dosage on the generation and expansion of HSCs in several hematopoietic sites throughout mouse development. We show that a haploid dose of *GATA-2* severely reduces production and expansion of HSCs specifically in the aorta-gonad-mesonephros region (which autonomously generates the first HSCs), whereas quantitative reduction of HSCs is minimal or unchanged in yolk sac, fetal liver, and adult bone marrow. However, HSCs in all these ontogenically distinct anatomical sites are qualitatively defective in serial or competitive transplantation assays. Also, cytotoxic drug-induced regeneration studies show a clear *GATA-2* dose-related proliferation defect in adult bone marrow. Thus, GATA-2 plays at least two functionally distinct roles during ontogeny of HSCs: the production and expansion of HSCs in the aorta-gonad-mesonephros and the proliferation of HSCs in the adult bone marrow.

Key words: GATA-2 • hematopoietic stem cells • AGM • haploinsufficiency • gene dosage

## Introduction

Hematopoietic stem cells (HSCs) at the foundation of the adult hematopoietic differentiation hierarchy have the ability to self-renew and produce all the distinct blood cell lineages (1, 2). HSCs capable of complete long-term hematopoietic repopulation of irradiated adult recipients are first generated in the aorta-gonads-mesonephros (AGM) region at mid-embryonic day (E)10.5 (3, 4) and localize to the dorsal aorta, vitelline, and umbilical arteries (5). Subsequently, at mid/late E11, HSC activity is also found in the yolk sac (YS) and fetal liver (FL). HSC activity increases significantly in E11 AGM and E12 YS when they are cultured as whole tissue explants for 3 d (3, 6). Although the spatial and temporal appearance of HSCs during development has been described and quantitated, the molecular mechanisms underlying HSC generation, expansion, and maintenance are not well explored.

One molecule important during hematopoietic ontogeny is GATA-2, a member of the GATA family of zinc finger transcription factors (7, 8). RT-PCR analysis shows high expression of *GATA-2* in adult hematopoietic progenitor cells and HSCs (9, 10). Furthermore, immunohistochemistry, in situ hybridization, and transgenic analyses show *GATA-2* expression as early as E8 in the para-aortic splanchnopleura (precursor tissue to AGM) and subsequently in the AGM (11, 12). In the E11.5 AGM, GATA-2 is expressed in the aortic endothelium and neighboring mesenchymal cells, which are both considered putative hemogenic cell populations. Embryos lacking GATA-2 are anemic, have moderately reduced numbers of primitive erythroid cells and he-

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Abbreviations used in this paper: AGM, aorta-gonads-mesonephros; CFU-GM, colony-forming unit-granulocyte macrophage; CFU-S<sub>11</sub>, colony-forming unit-spleen; E, embryonic day; ER, estrogen receptor; ES, embryonic stem; FL, fetal liver; 5-FU, 5-fluorouracil; GFP, green fluorescent protein; HSC, hematopoietic stem cell; LSK, Lin<sup>-</sup>Sca-1<sup>+</sup>c-kit<sup>+</sup>; YS, yolk sac.

matopoietic progenitor cells (13), and die at E10.5, the time of HSC induction and expansion. Due to this lethality, the role of GATA-2 has been examined mainly by in vitro colony-forming assays of cells from early embryonic tissues and hematopoietic cultures of *GATA-2*<sup>-/-</sup> embryonic stem (ES) cells. In both cases, hematopoietic progenitor numbers are severely reduced. Further studies in chimeric mice produced with *GATA-2*<sup>-/-</sup> ES cells show no contribution of the mutant cells to any hematopoietic tissues. Together, these data indicate that GATA-2 is crucial for the maintenance, proliferation, and/or survival of immature hematopoietic progenitors (13).

The function of GATA-2 had also been studied through enforced overexpression achieved by retroviral transduction or transfection of genes encoding either a wild-type GATA-2 or an inducible GATA-2–estrogen receptor (ER) fusion protein (12, 14–19). Irrespective of these attempts, a simple conclusion on the function of GATA-2 in the HSCs or progenitor cells is difficult. On one hand, expression of the inducible form of GATA-2–ER fusion protein in the multipotent hematopoietic progenitor cell line FDCP promotes differentiation (17). On the other hand, constitutive expression of GATA-2 in murine BM cells blocked progenitor-derived colony formation (14). The opposing results may be due to the cell types chosen for these experiments. However, it is also suggested that the biochemical behavior of the artificially generated GATA-2–ER fusion protein may not be the same as the wild-type unmodified GATA-2 protein (18). Thus, the most relevant data on *GATA-2* dose effects on hematopoiesis may be best obtained within the physiological context of the whole organism wherein *GATA-2* is expressed under the endogenous regulatory machinery in the appropriate cell types.

The study of transcription factor dose and function at the earliest stages of hematopoietic development is of particular interest for an understanding of HSC generation. Studies on the *runx1* transcription factor have shown that a haploid dose results in changes in HSC induction, expansion, and distribution in the midgestation mouse embryo (20). Moreover, haploinsufficiency of human *Runx1* (AML-1) results in thrombocytopenia and a propensity to develop myeloid leukemia (21). Recently, a correlation between a reduction in *GATA-2* expression and aplastic anemia (22) has been demonstrated. Hence, to further understand the role of GATA-2 in the ontogeny of HSCs we examined the effects of *GATA-2* haploinsufficiency on induction and expansion of HSCs during development by in vivo hematopoietic transplantation assays and phenotypic analysis of compound transgenic embryos (*GATA-2*<sup>+/-</sup>–*Ly-6A GFP*) (23).

Here we present data showing that the numbers of hematopoietic progenitors in *GATA-2*<sup>+/-</sup> embryos are reduced. More importantly, we observe a dramatic quantitative reduction in HSC activity specifically in *GATA-2*<sup>+/-</sup> AGMs and a further reduction in the serial repopulating ability of these HSCs. In contrast, *GATA-2*<sup>+/-</sup> HSC numbers appear quantitatively normal in the adult BM but are qualitatively defective in the setting of competitive transplantation. In addition, *GATA-2*<sup>+/-</sup> HSCs exhibit a delay

in regeneration of the hematopoietic system after cytotoxic drug challenge, suggesting that GATA-2 levels play a role in HSC proliferation. Thus, GATA-2 plays functionally distinct roles in the production of HSCs in the AGM region and the proliferation of HSCs throughout ontogeny.

## Materials and Methods

***GATA-2 Mutant Mice and Embryos.*** *GATA-2* mutant mice (13) were backcrossed onto the C57BL/6 background for over 10 generations and were housed in the Erasmus Medical Center Animal unit according to the institution guidelines with food and water provided ad libitum. The day of vaginal plug discovery from overnight matings (*GATA-2*<sup>+/-</sup> male × C57BL/6 *GATA-2*<sup>+/+</sup> or *GATA-2*<sup>+/-</sup> female) was counted as day 0. Pregnant dams were killed and embryos isolated from the uterus as described previously (24). Embryos (E10–E11) were staged by counting somite pairs (25). Genotyping was performed by PCR as described previously (13). Compound transgenic embryos were obtained by mating *Ly-6A GFP* hemizygous (23) and *GATA-2*<sup>+/-</sup> mice.

***Dissection, Explant Culture, Cell Preparation, and In Vivo Transplantation.*** Dissections, tissue explants, and cell preparation were performed as described previously (24). Recipient mice (C57BL/6 or [129Sv × C57BL/6] F1 females, 8–16 wk old) received a split dose of 1,000 rad (for colony-forming unit–spleen [CFU-S<sub>11</sub>]), 900 rad (for HSCs), or 640 rad (for competitive repopulation assay) at a 3-h interval from a <sup>137</sup>Cs source on the day of donor cell injection. Cells were injected i.v. into the tail veins. Except for CFU-S, serial, and competitive transplantation assays, 2 × 10<sup>5</sup> female spleen cells from the recipient strain were coinjected to provide short-term survival. Secondary transplantations were performed with 3 × 10<sup>6</sup> BM cells from the primary recipients. Cell dose for competitive repopulation assays was 3 × 10<sup>5</sup>–3 × 10<sup>7</sup>. Injected animals were provided with 0.16% Neomycin (Sigma-Aldrich)-supplemented water. For CFU-S<sub>11</sub>, recipients were killed at 11 d posttransplantation by cervical dislocation, spleens isolated, and microscopic colonies scored after fixing with Teley-niesnicki's solution overnight.

***Semiquantitative PCR for Donor Contribution.*** Blood, tissue, or specific cell lineage DNA (100 ng) was used for semiquantitative PCR to detect the donor HSC contribution to the recipient. For male-derived donor cells, *YMT*-specific PCR (350-bp product) was used together with myogenin-specific PCR (250 bp) for DNA normalization. The detection of *GATA-2* mutant–derived donor cells was performed with *GATA-2/NEO* (950 bp) and *GATA-2* wild-type (600 bp) PCR. Primers and PCR conditions were as previously described (4, 13).

***Cell Sorting and Flow Cytometry Analysis.*** FACS was performed on a FACS Vantage SE (Becton Dickinson) (23), and flow cytometric analyses were performed on a FACSCalibur dual laser instrument (Becton Dickinson) with CellQuest software (BD Bioscience). Staining of embryonic tissue cell suspensions was performed in PBS supplemented with 10% FCS, and 2 μg/ml 7AAD (Molecular Probes) was added for dead cell exclusion. Staining of adult HSCs was performed in PBS supplemented with 0.5% BSA. Biotin-conjugated anti-Gr-1 (ER-MP20) was a gift from Dr. P.J.M. Leenen (Erasmus MC, Rotterdam, The Netherlands). All other antibodies were obtained from BD Biosciences including APC-conjugated anti-c-kit (clone 2B8), PE-conjugated anti-Sca-1 (clone D7), PerCP-Cy5.5-conjugated anti-CD8a (clone 53–6.7), anti-B220 (clone RA3-6B2), anti-CD19

(1D3), anti-CD11b (anti-Mac-1, clone M1/70), and biotin-conjugated anti-CD3 (145-2C11), anti-CD4 (H129.19), Ly-76 (TER-119), anti-IgM (II/41), and anti-NK1.1 (PK136). A secondary step was sometimes performed with streptavidin-conjugated PerCP-Cy5.5 (BD Biosciences).

**Immunohistochemistry.** Embryos were fixed for 30 min with 2% paraformaldehyde/PBS at 4°C and equilibrated in 20% sucrose/PBS overnight at 4°C. They were immersed in Tissue Tek, quick frozen on dry ice, and stored at -80°C until ready for use. Serial cryosections (10 µm) were treated in 100% cold acetone for 10 min, washed three times with PBS (0.05% Tween), blocked with 0.5% BSA and 50% vol/vol Avidin D block solution (Vector Laboratories) for 15 min, washed three times, blocked with Biotin blocking solution (Vector Laboratories) for 15 min, and washed three times. Subsequently, sections were incubated with a biotin-conjugated anti-CD34 (clone RAM34; BD Biosciences) diluted 1:50 in 1% BSA/0.05% Tween/PBS at room temperature for 1 h, washed three times, incubated with the detection reagent Streptavidin-Cy5 (Rockland) diluted 1:500 in 1% BSA/0.05% Tween/PBS at room temperature for 30 min, washed three times, dehydrated in ethanol (from 70 to 100%), and mounted with vectashield (Vector Laboratories).

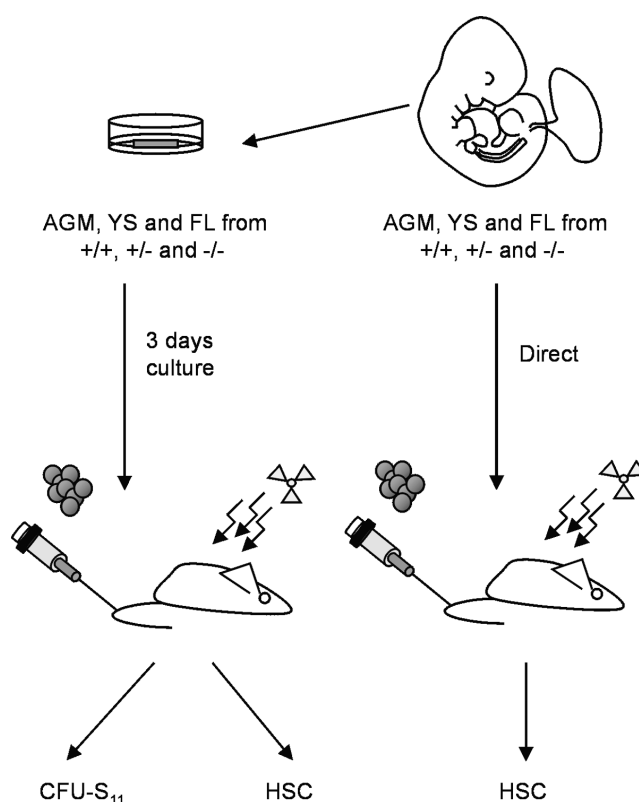
Confocal images were taken of every tenth section starting caudally at the point where the urogenital ridges first appeared up to the rostral bifurcation of the dorsal aorta.

**5-Fluorouracil Treatment.** *GATA-2*<sup>+/+</sup> and *GATA-2*<sup>+/-</sup> mice (9–10 wk old) were i.v. injected with 150 mg 5-fluorouracil (5-FU; Sigma-Aldrich) per 10 g body weight. Treated mice were then killed at 4, 8, 12, and 16 d posttreatment, and BM cells were isolated and analyzed by flow cytometry and in vitro culture assay.

**Progenitor Colony Assay.** BM cells were plated in triplicates from  $2 \times 10^4$  to  $5 \times 10^5$  cells per plate in methylcellulose medium (Methocult GF M3434; StemCell Technologies Inc.) supplemented with stem cell factor (SCF), IL-3, IL-6, and Epo. All cultures were incubated at 37°C in a humidified chamber under 5% CO<sub>2</sub>. Colony-forming unit-granulocyte macrophage (CFU-GM) were scored with an inverted microscope at day 7 of the culture.

## Results

***GATA-2*<sup>+/-</sup> AGM and YS Explants Contain Fewer CFU-S<sub>11</sub>.** To investigate if *GATA-2* gene dosage affects the production of hematopoietic progenitors during development, colony-forming unit spleen activity was assayed at 11 d postinjection (CFU-S<sub>11</sub>) so as to measure the more immature erythro-myeloid progenitors. AGM, YS, and FL were isolated from *GATA-2*<sup>+/+</sup> and *GATA-2*<sup>+/-</sup> embryos at E10.5–E12, explant cultured, and cells transplanted into irradiated adult recipients (Fig. 1). At all time points tested, CFU-S<sub>11</sub> activity was detected both in *GATA-2*<sup>+/+</sup> and *GATA-2*<sup>+/-</sup> AGM, YS, and FL explants (Fig. 2). As expected from previous data (3), high numbers of E10.5 CFU-S<sub>11</sub> are first detected in the *GATA-2*<sup>+/+</sup> AGM explants, they increase at E11, and thereafter decline in number. In *GATA-2*<sup>+/-</sup> AGM and YS explants, CFU-S<sub>11</sub> were reduced by three- to ninefold and one- to fourfold, respectively, compared with wild-type tissues. In contrast, CFU-S<sub>11</sub> activity in *GATA-2*<sup>+/-</sup> FL explants was normal. No reductions in FL CFU-S<sub>11</sub> numbers were observed at any time. Therefore, *GATA-2* gene dosage affects the generation and/or proliferation of immature hemato-

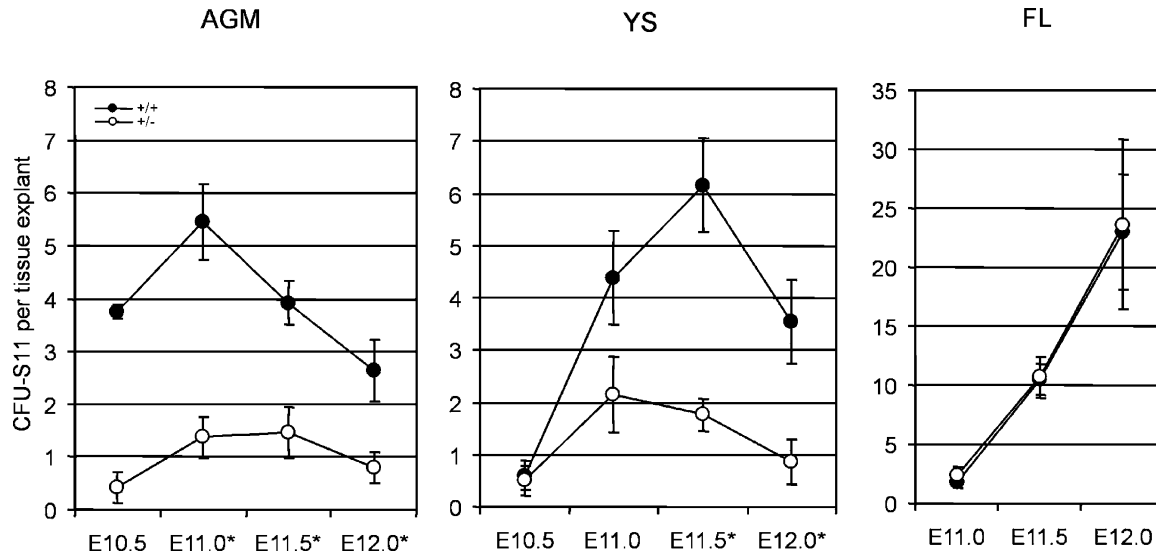


**Figure 1.** Strategy for studying HSCs and hematopoietic progenitor cells in *GATA-2* mutant embryonic tissues. *GATA-2*<sup>+/+</sup>, *GATA-2*<sup>+/-</sup>, and *GATA-2*<sup>-/-</sup> AGM regions, YS, and FL were harvested from mid-gestation mouse embryos. In some cases (left) they were then cultured for 3 d as whole tissue explants before preparation of single cell suspensions and injection into irradiated adult recipients to assay for CFU-S<sub>11</sub> or HSCs. In some cases (right), single cell suspensions were prepared directly from freshly isolated tissues and injected into irradiated adult recipients for HSC activity.

etic progenitor cells in the YS and AGM of the mid-gestation embryo.

**HSC Activity Is Severely Reduced in the *GATA-2*<sup>+/-</sup> AGMs.** To investigate if *GATA-2* dose also affects mid-gestation HSC development, we performed the most stringent functional HSC test: the long-term, high level, multilineage repopulation of irradiated adult mouse recipients. E11 and E12 *GATA-2*<sup>+/+</sup> and *GATA-2*<sup>+/-</sup> AGM, YS, and FL cells were transplanted directly into irradiated adult recipients (Fig. 1). Engraftment by *GATA-2*<sup>+/+</sup> and *GATA-2*<sup>+/-</sup> cells was assayed by semiquantitative PCR of the male Y chromosome-specific marker *Ymt* and the *GATA-2*/*NEO* mutant allele (respectively) in recipient peripheral blood DNA at 4 mo posttransplantation. Only those recipients showing >10% engraftment with donor-marked cells were considered positive for high level repopulation. PCR results of one representative experiment are shown in Fig. 3. Briefly, each recipient received one-third of the cells obtained from an individually prepared E12 tissue (0.33 tissue equivalents). At 4 mo postinjection, progeny of transplanted *GATA-2*<sup>+/+</sup> AGM cells were found in the peripheral blood of five out of eight recipients (*Ymt*



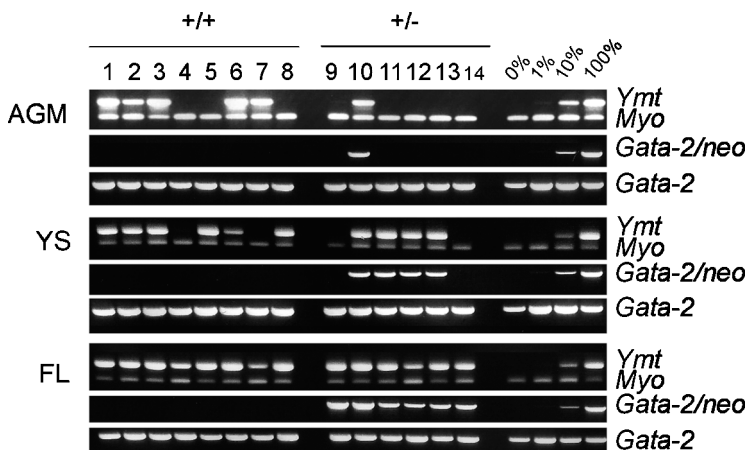


**Figure 2.** CFU-S<sub>11</sub> activity in *GATA-2* mutant tissue explants. CFU-S<sub>11</sub> were assayed from AGM, YS, and FL explants (E10.5–E12.0) after 3 d of culture. Each point represents the average CFU-S<sub>11</sub> number per embryo tissue equivalent  $\pm$  SEM detected in the corresponding tissue and genotype. 3–14 independent experiments were performed with 0.2–4.5 tissue equivalents injected per recipient. +/+, *GATA-2*<sup>+/+</sup>; +/-, *GATA-2*<sup>+/-</sup>. The total number of injected AGM explants: E10.5 +/+ = 6, E11.0 +/+ = 21, E11.5 +/+ = 14, E12.0 +/+ = 12, E10.5 +/- = 7, E11.0 +/- = 22, E11.5 +/- = 12, E12.0 +/- = 12; YS explants E10.5 +/+ = 8, E11.0 +/+ = 8, E11.5 +/+ = 11, E12.0 +/+ = 10.75, E10.5 +/- = 8, E11.0 +/- = 11.5, E11.5 +/- = 15.5, E12.0 +/- = 9.5; FL explants E11.0 +/+ = 17, E11.5 +/+ = 18, E12.0 +/+ = 8.7, E11.0 +/- = 23, E11.5 +/- = 19.5, E12.0 +/- = 8. Embryonic tissues from E10.5 ranged from 36 to 40 somite pairs (sp), E11.0 ranged from 41–47 sp, E11.5 contain >48 sp and E12 contained >60 sp. \*Significant difference in the CFU-S<sub>11</sub> number between *GATA-2*<sup>+/+</sup> and *GATA-2*<sup>+/-</sup> tissue explants: AGM E11.0, *P* < 0.001; E11.5, *P* < 0.01; E12.0, *P* < 0.05; YS E11.5, *P* < 0.001; E12, *P* < 0.05. Note that fewer CFU-S<sub>11</sub> are detected in both the *GATA-2*<sup>+/-</sup> AGM and YS explants in comparison to the *GATA-2*<sup>+/+</sup> explants, whereas the FL CFU-S<sub>11</sub> numbers are unaffected.

PCR). In contrast, reduced HSC activity was found in E12 *GATA-2*<sup>+/-</sup> AGMs. Only one out of six recipients was highly engrafted (*GATA-2* mutant and *Ymt* PCR). Similarly, recipients transplanted with YS cells revealed some reduction of HSC activity in *GATA-2*<sup>+/-</sup> embryos. However, no reduction in HSC activity was found in *GATA-2*<sup>+/-</sup> FLs. Further analysis of recipients repopulated with *GATA-2*<sup>+/-</sup> cells revealed high level multilineage engraftment within all hematopoietic tissues (blood, thymus, LNs, BM, and spleen) and subsets tested (splenic T and B lymphocytes, erythroid and myeloid cells) (not depicted).

The cumulative results of all transplantation experiments are shown in Table I and reveal that at both E11 and E12,

HSC activity is severely reduced in *GATA-2*<sup>+/-</sup> AGMs. The percentage of recipients repopulated with E11 *GATA-2*<sup>+/-</sup> AGM cells is only 6%, whereas 25% of recipients are repopulated with E11 *GATA-2*<sup>+/+</sup> AGM cells. This represents a greater than fourfold decrease in HSC activity in E11 *GATA-2*<sup>+/-</sup> AGMs. Furthermore, at E12 *GATA-2*<sup>+/-</sup> AGMs are ninefold reduced in HSC activity compared with *GATA-2*<sup>+/+</sup> AGMs. Reductions in the HSC activity of *GATA-2*<sup>+/-</sup> YS and FL tissues are less severe and stage dependent. The percentage of mice repopulated by E11 *GATA-2*<sup>+/-</sup> YS (23%) is comparable to that of *GATA-2*<sup>+/+</sup> YS (28%), and the FL at this stage contains only limited HSC activity. However, at E12 slight reductions in HSC



**Figure 3.** Detection of donor hematopoietic cell contribution in transplantation recipients by peripheral blood DNA PCR analysis. A representative PCR analysis for donor cell contribution to the peripheral blood of transplantation recipients. DNA was isolated from the corresponding recipients (at >4 mo posttransplantation) of *GATA-2*<sup>+/+</sup>, *GATA-2*<sup>+/-</sup>, and *GATA-2*<sup>-/-</sup> AGM, YS, and FL. Lanes 1–8 and 9–14 are blood DNA samples isolated from recipients receiving cells from E12 *GATA-2*<sup>+/+</sup> and *GATA-2*<sup>+/-</sup> tissues, respectively. Each sample was analyzed with primers specific for Y chromosome (*ymt*), and *GATA-2* (*GATA-2/NEO* for targeted allele). DNA samples were normalized by PCR with two endogenous gene controls (*myo*, *myogenin*; *GATA-2*, wild-type allele). Control DNA: 0, 1, 10, and 100% represents percentage of the male *GATA-2*<sup>+/-</sup> DNA mixed with female DNA. Only when the donor marker-specific PCR product was >10%, compared with controls, was the recipient considered to be positive.

**Table I.** HSC Activity in *GATA-2* Mutant Embryonic Tissues

Stage	AGM		YS		FL	
	+/+	+/-	+/+	+/-	+/+	+/-
E11.0	1 <sup>a</sup> /4 <sup>b</sup> (25) <sup>c</sup>	1/16 (6)	2/7 (28)	4/17 (23)	0/6 (0)	1/19 (5)
E12.0	11/16 (69)	1/13 (8)	11/15 (73)	6/13 (46)	19/19 (100)	11/14 (78)
2 <sup>o</sup> E12.0	6/6 (100)	0/3 (0)	6/6 (100)	4/9 (44)	6/6 (100)	4/9 (44)

E11 and E12 AGM, YS, and FL tissues were made into a single cell suspension and injected into irradiated adult recipients. Each result represents <sup>a</sup>the number of recipient mice showing donor cells in peripheral blood (DNA) at >4 mo posttransplantation, <sup>b</sup>the total number of mice transplanted, and <sup>c</sup>the percentage of repopulated recipients. Only when the donor cells represented >10% was the recipient considered to be positive. Three and two independent experiments, respectively, were performed for E11 (41–47 somite pairs) and E12 tissues (>60 somite pairs). 1 and 0.33 tissue equivalents transplanted for E11.0 and E12.0, respectively. 2<sup>o</sup>E12, secondary transplantation with  $3 \times 10^6$  BM cells isolated from high level repopulated primary recipients that received cells from E12 tissues (two independent experiments). +/+, *GATA-2*<sup>+/+</sup>; +/-, *GATA-2*<sup>+/-</sup>.

activity are observed for both *GATA-2*<sup>+/-</sup> YS and FL (1.6- and 1.3-fold, respectively) compared with *GATA-2*<sup>+/+</sup> tissues. Thus, two copies of the *GATA-2* gene are required for the normal generation, expansion, and/or survival of HSCs in the AGM region.

*Ex Vivo Expansion and Maintenance of AGM HSC Activity Is Sensitive to GATA-2 Dose.* Since it was shown previously that HSC activity generated in the AGM can be amplified (either by induction or proliferation) when whole tissues are cultured for 3 d (3), we examined the effects of *GATA-2* gene dosage on HSCs in such explant cultures of AGM, YS, and FL from *GATA-2*<sup>+/+</sup> and *GATA-2*<sup>+/-</sup> embryos. Tissues (E10.5–E12) were dissected, cultured as explants for 3 d, made into a single cell suspension, and injected into irradiated adult recipients (Fig. 1). Repopulation was measured at 4 mo posttransplantation, and only those recipients showing >10% donor cell multilineage hematopoietic repopulation (measured in several hematopoi-

etic tissues and lineages) were considered positive. The results are summarized in Table II.

Compared with the results of the direct transplantation experiments (Table I), *GATA-2*<sup>+/-</sup> AGM explants were even more severely reduced in the HSC activity. At E10.5, only 8% of recipients receiving *GATA-2*<sup>+/-</sup> AGM cells were repopulated, representing an eightfold decrease in HSC activity from *GATA-2*<sup>+/+</sup> AGM cells. The *GATA-2*<sup>+/-</sup> cell contribution to the various hematopoietic organs (thymus, spleen, LN, and BM) and purified cell lineages (B and T lymphocytes, myeloid and erythroid) was tested and found to be multipotent, thus demonstrating that *GATA-2*<sup>+/-</sup> AGMs do generate functional HSCs, albeit at much reduced levels. At later developmental time points (E11, E11.5, and E12), HSC activity, although increasing in *GATA-2*<sup>+/+</sup> AGM explants, is completely absent from *GATA-2*<sup>+/-</sup> AGM explants. As seen in the *GATA-2*<sup>+/+</sup> AGM explants, HSC generation and expansion occurs from

**Table II.** HSC Activity in *GATA-2* Mutant Tissues after Explant Culture

Stage	AGM explants			YS explants			FL explants	
	+/+	+/-		+/+	+/-		+/+	+/-
E10.5	2 <sup>a</sup> /3 <sup>b</sup> (66) <sup>c</sup>	1/12 (8)		0/3 (0)	0/11 (0)		ND	0/2 (0)
E11.0	1/2 (50)	0/4 (0)		1/4 (25)	1/5 (20)		0/3 (0)	0/5 (0)
E11.5	11/12 (50)	0/27 (0)		7/25 (28)	7/29 (24)		6/25 (24)	3/28 (11)
E12.0	7/12 (58)	0/13 (0)		4/16 (25)	2/14 (14)		11/12 (92)	10/19 (53)
E10.0	+/+	+/-	-/-	+/+	+/-	-/-	+/+	+/-
	0/7 (0)	0/8 (0)	0/3 (0)	0/7 (0)	0/8 (0)	0/3 (0)	ND	ND

E10 to E12 AGM, YS, and FL explants were cultured for 3 d, made into a single cell suspension, and injected into irradiated adult recipients. Each result represents <sup>a</sup>the number of recipient mice showing donor cells in the peripheral blood (DNA) isolated at >4 mo posttransplantation, <sup>b</sup>the total number of mice transplanted, and <sup>c</sup>the percentage of repopulated recipients. ND, transplantation not performed. Only when donor contribution was >10% was the recipient considered to be positive. Two to four independent experiments performed for each stage and tissue type. For E10, one to five tissue explant equivalents were transplanted per recipient. 1 tissue explant equivalent was transplanted per recipient for E10.5 and 0.33 tissue explant equivalents for E11.0, E11.5, and E12.0. +/+, *GATA-2*<sup>+/+</sup> and +/-, *GATA-2*<sup>+/-</sup>. E10, 31–35 somite pairs (sp); E10.5, 36–40 sp; E11.0, 41–47 sp; E11.5 > 48 sp; E12.0 > 60 sp.

E10.5 to E11.5, with HSC numbers maintained thereafter (E12). Thus, the severely reduced HSC activity in *GATA-2*<sup>+/-</sup> AGM explants can be attributed to reduced HSC expansion, survival, and/or homing in the irradiated recipient.

The HSC activity of *GATA-2*<sup>+/-</sup> YS and FL explants was also reduced in comparison to the *GATA-2*<sup>+/+</sup> explants. However, this reduction was only slight compared with the AGM. At the first appearance of HSCs in the YS on E11, *GATA-2*<sup>+/-</sup> YS HSC activity begins to decrease and by E12 is decreased by 1.8-fold from *GATA-2*<sup>+/+</sup> YS. The decrease in YS explant HSC activity at E12 corresponds with that seen in the directly transplanted YS, suggesting that the expansion but not the maintenance of YS HSC activity is sensitive to *GATA-2* dose.

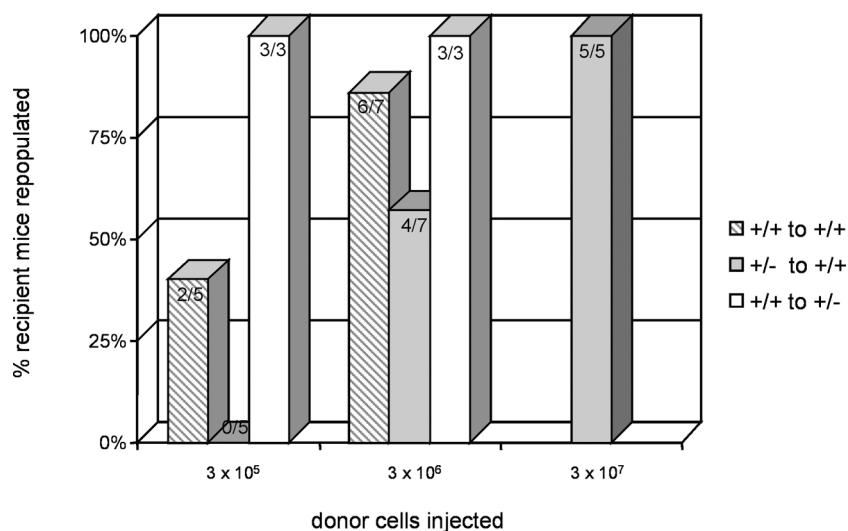
Similarly, we observed slight reductions in the HSC activity of *GATA-2*<sup>+/-</sup> FL explants. At the first appearance of HSCs in FL explants on E11.5, HSC activity is reduced from 24% of recipients repopulated with *GATA-2*<sup>+/+</sup> cells to 11% repopulated with *GATA-2*<sup>+/-</sup> cells, representing a 2.2-fold decrease in HSC activity. At E12, *GATA-2*<sup>+/-</sup> FL explants show a 1.7-fold decrease in HSC activity. The changes in FL HSC activity with time are most likely related to the numbers of incoming HSCs from the AGM and YS. Thus, these findings suggest *GATA-2* dose affects the expansion but not the survival of HSCs in the FL.

*The Onset of HSC Activity in GATA-2*<sup>+/-</sup> *Embryos Is Normal.* Previously, we reported CFU-S<sub>11</sub> and HSC deficiencies in embryos with a haploid dose of the *runx1* transcription factor (20). The spatial distribution of HSC activity was altered and an unexpected early appearance of HSC activity was found in *runx1*<sup>1+/-</sup> AGM and YS. To examine if there was also a premature appearance of HSC activity in *GATA-2* mutant embryos, AGM and YS explants from early E10 (31–35 somite pairs) *GATA-2*<sup>+/+</sup>, *GATA-2*<sup>+/-</sup>, and *GATA-2*<sup>-/-</sup> embryos were isolated, cultured for 3 d, and cells were transplanted into irradiated adult recipients. As shown in Table II, although high tissue equivalents (up to five) of cells from *GATA-2* mutant (+/- and -/-) AGM and YS explants were injected, HSC activity was not

detected in any of the recipients. Also, *GATA-2*<sup>+/-</sup> E10.5 YS and E10.5 and E11 FL explants showed no HSC activity. However, HSC activity initiates normally in *GATA-2*<sup>+/-</sup> AGM explants at E10.5 at the same stage as in the *GATA-2*<sup>+/+</sup> AGM. HSCs also appear at normal time points in *GATA-2*<sup>+/-</sup> YS and FL explants (E11 and E11.5, respectively). Therefore, we conclude that HSC induction initiates on schedule and that there is no early onset of HSC activity in *GATA-2*<sup>+/-</sup> AGM, YS, or FL.

*Serial Transplantation Potential of Midgestation HSCs Is Severely Reduced.* HSC self-renewal can be tested by serial transplantation of HSCs from primary to secondary recipients. Since we found that *GATA-2*<sup>+/-</sup> AGM HSCs are severely reduced in their expansion, we examined whether *GATA-2*<sup>+/-</sup> embryo-derived HSCs are as potent in their serial repopulation ability as wild-type HSCs. Whole BM cells from primary recipient mice showing high donor contribution from transplanted E12 *GATA-2*<sup>+/+</sup> or *GATA-2*<sup>+/-</sup> AGM, YS, and FL cells were injected into irradiated secondary adult recipients. Consistent with previous published results, *GATA-2*<sup>+/+</sup> AGM-, YS-, and FL-derived HSCs can successfully reconstitute secondary recipients; 100% of secondary recipients were repopulated with HSCs from primary recipients of these midgestation tissues (Table I). In contrast, HSCs from a primary *GATA-2*<sup>+/-</sup> AGM recipient failed to repopulate any of the secondary recipients analyzed (0%; zero out of three). Reduced HSC activity was also observed in the secondary recipients receiving BM cells from *GATA-2*<sup>+/-</sup> YS and FL primary recipients (44% compared with 100% recipient repopulation with *GATA-2*<sup>+/+</sup> primary BM cells). These results demonstrate that *GATA-2* dose affects HSC serial repopulation ability and suggests a defect in HSC self-renewal.

*GATA-2*<sup>+/-</sup> *BM HSCs Are at a Competitive Disadvantage.* The decreased serial repopulation ability of embryo-derived HSCs prompted us to investigate if adult BM HSCs are also affected by a reduction in *GATA-2* dose. Initially, we injected limiting doses of *GATA-2*<sup>+/+</sup> and *GATA-2*<sup>+/-</sup> BM cells into lethally irradiated adult recipients but found



**Figure 4.** Competitive transplantation of *GATA-2*<sup>+/+</sup> and *GATA-2*<sup>+/-</sup> BM in sublethally irradiated adult recipients. Varying concentrations ( $3 \times 10^5$ – $3 \times 10^7$ ) of *GATA-2*<sup>+/+</sup> or *GATA-2*<sup>+/-</sup> BM cells were transplanted into sublethally irradiated *GATA-2*<sup>+/+</sup> or *GATA-2*<sup>+/-</sup> recipients to test for HSC competition in repopulation. The y axis shows the percentage of recipient animals engrafted with >10% donor cells in hematopoietic tissues. Engraftment results are shown in gray striped bars for *GATA-2*<sup>+/+</sup> donor cells transplanted into *GATA-2*<sup>+/+</sup> recipients, in gray bars for *GATA-2*<sup>+/-</sup> donor cells transplanted into *GATA-2*<sup>+/+</sup> recipients, and in white bars for *GATA-2*<sup>+/+</sup> donor cells transplanted into *GATA-2*<sup>+/-</sup>. The results show that *GATA-2*<sup>+/+</sup> HSCs out-compete *GATA-2*<sup>+/-</sup> HSCs.

no quantitative differences in repopulation. Hence, we performed reciprocal competitive transplantations in which different concentrations of unmanipulated  $GATA-2^{+/+}$  and  $GATA-2^{+/-}$  BM cells were injected into sublethally irradiated  $GATA-2^{+/+}$  and  $GATA-2^{+/-}$  adult recipients.

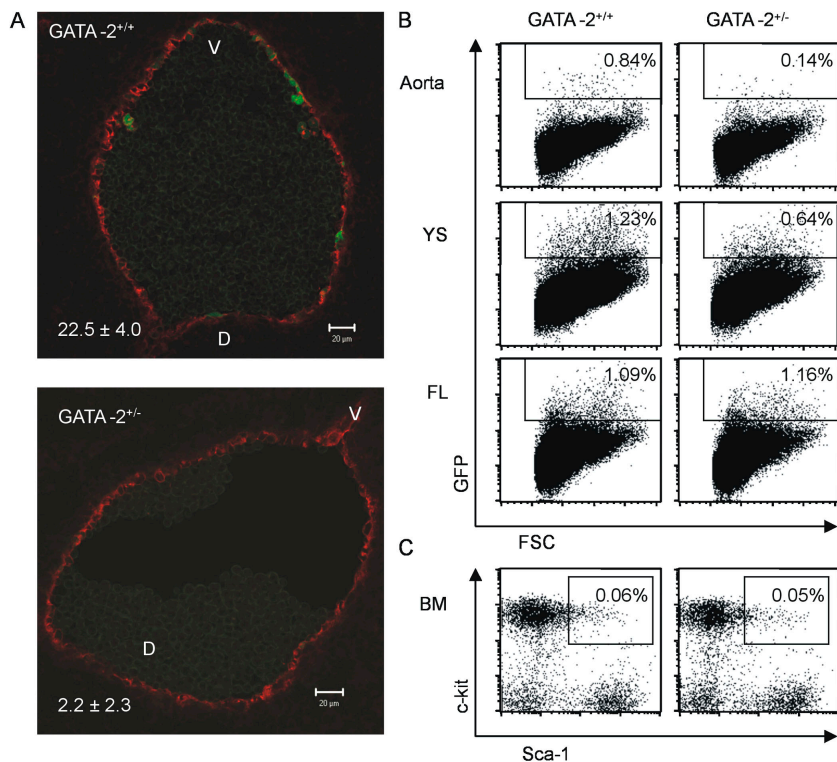
When  $3 \times 10^5$  whole  $GATA-2^{+/+}$  BM cells were transplanted into  $GATA-2^{+/+}$  adult recipients, long-term high level donor contribution was found in two out of five (40%) recipients, whereas  $GATA-2^{+/-}$  cells at this dose provided no repopulation (zero out of five recipients; 0%) (Fig. 4). Only at a 10-fold higher cell dose were the  $GATA-2^{+/-}$  cells able to repopulate four out of seven (57%) recipients. A dose of  $3 \times 10^6$   $GATA-2^{+/+}$  cells repopulated almost all recipients (six out of seven; 86%), whereas  $3 \times 10^7$   $GATA-2^{+/-}$  cells were required for repopulation of all recipients (five out of five; 100%). To further examine the competitive abilities of  $GATA-2^{+/-}$  cells,  $GATA-2^{+/+}$  cells were injected into sublethally irradiated  $GATA-2^{+/-}$  recipients. Only  $3 \times 10^5$  (or fewer) wild-type cells were required to fully out-compete all the  $GATA-2^{+/-}$  HSCs in the recipients. Thus,  $GATA-2^{+/+}$  HSCs compete more effectively against  $GATA-2^{+/-}$  HSCs than do  $GATA-2^{+/-}$  HSCs, demonstrating that  $GATA-2^{+/-}$  adult BM contains fewer HSCs or that these HSCs are qualitatively less potent.

***GATA-2 Dose Affects the Number of Phenotypically Defined HSCs in the Embryo But Not the Adult.*** Our *in vivo* transplantation results clearly show that  $GATA-2^{+/-}$  HSC activity is affected throughout development. To more specifically investigate the cell types that are affected in the  $GATA-2^{+/-}$  mice, we crossed the  $GATA-2$  mutant allele

into  $Ly-6A$  GFP transgenic mice, in which HSCs can be detected by the expression of the green fluorescent protein (GFP) reporter under the transcriptional control of  $Ly-6A$  regulatory sequences (26).  $Ly-6A$  encodes the Sca-1 surface glycoprotein that is expressed on HSCs. Previously, we have shown that all AGM, FL, and adult BM HSCs express the  $Ly-6A$  GFP transgene and that GFP expression is highly restricted in the AGM region to a few aortic endothelial cells and hematopoietic clusters (23, 27).

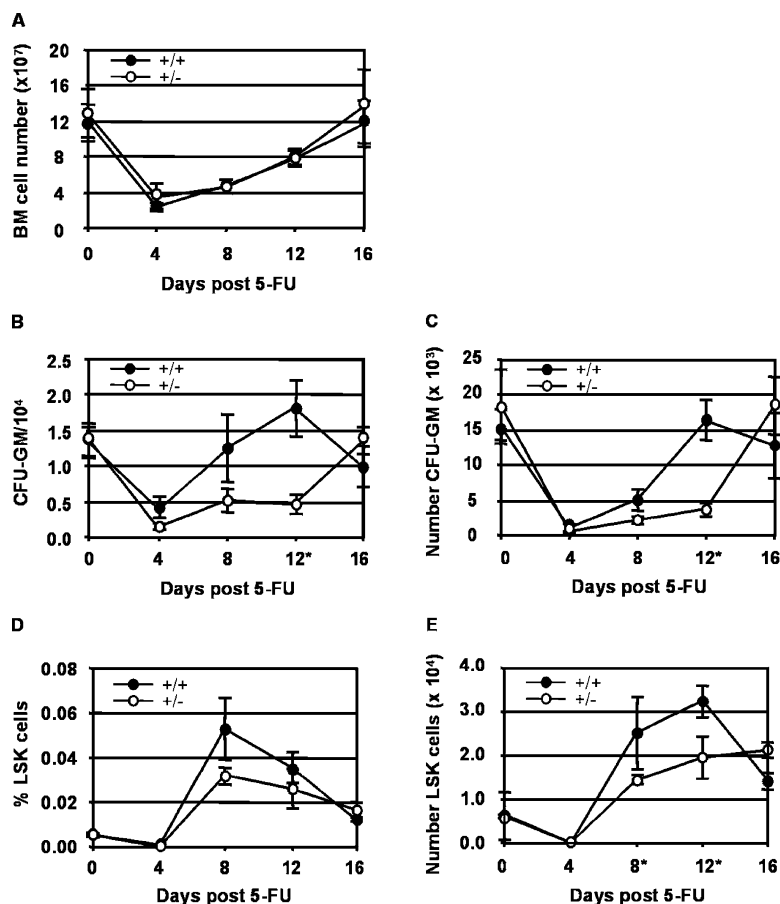
To determine whether  $GATA-2$  dose affects these cells, we examined transverse sections through the E11 dorsal aorta from compound transgenics ( $Ly-6A$  GFP- $GATA-2^{+/+}$  and  $Ly-6A$  GFP- $GATA-2^{+/-}$ ). As shown in representative sections in Fig. 5 A, GFP-positive cells are decreased in number in the  $GATA-2^{+/-}$  aorta compared with the  $GATA-2^{+/+}$  aorta. Quantitations were performed by counting GFP-positive and CD34-positive cells in 37 aorta sections from each genotype (CD34 immunostaining of endothelial cells provided a normalization control). GFP-positive cells were present but decreased by a factor of 10 or more in the  $GATA-2^{+/-}$  aortas. Hence,  $GATA-2$  haploinsufficiency leads to a significant decrease in HSCs and/or HSC precursors in the AGM.

Flow cytometric analyses were performed to verify these results and to examine if the phenotypic HSC content of the other hematopoietic tissues was also changed. As shown in Fig. 5 B, in such compound transgenic embryos a sixfold decrease in GFP-positive cells in the E11  $GATA-2^{+/-}$  aorta was found compared with  $GATA-2^{+/+}$  aorta. E11  $GATA-2^{+/-}$  YS showed a 1.9-fold decrease in GFP-positive cells. However, no decrease was found in E11  $GATA-$



**Figure 5.** Phenotypic analysis of HSCs in  $GATA-2^{+/-}$  embryos and adults.  $GATA-2$ - $Ly-6A$  GFP compound transgenic embryos were generated by timed pluggings. (A) Representative transverse sections through the E11 dorsal aorta of a  $GATA-2^{+/+}$ - $Ly-6A$  GFP embryo (45 somite pairs; top) and a  $GATA-2^{+/-}$ - $Ly-6A$  GFP embryo (43 somite pairs; bottom). Sections were taken from the caudal end of the AGM, at the height of the hindgut, and stained with anti-CD34 antibody. In total, four embryos were analyzed (2 embryos and a total of 37 sections from each genotype) and cells counted in the aortic endothelium throughout the levels containing the gonads and mesonephroi. CD34<sup>+</sup> endothelial cells served as a control for section quality and normalization. Red fluorescence (CD34) and green fluorescence (GFP). The percentage of GFP<sup>+</sup>/CD34<sup>+</sup> endothelial cells  $\pm$  SEM is shown on the bottom left and is significantly reduced in the  $GATA-2^{+/-}$  embryos;  $P < 0.05$ . Flow cytometric analysis of phenotypically defined HSCs was performed on (B) embryonic hematopoietic tissues and (C) adult BM. Expression of the  $Ly-6A$  GFP HSC marker was analyzed on E11 aorta, YS, and FL cells. Adult BM cells were analyzed for the percentage of cells in the Lin<sup>-</sup> fraction that are Sca-1<sup>+</sup>c-kit<sup>+</sup>. Percentages of GFP<sup>+</sup> cells in the embryonic tissue and Sca-1<sup>+</sup>c-kit<sup>+</sup> cells enclosed in each gate are shown.





**Figure 6.** Hematopoietic regeneration after 5-FU treatment. The temporal regeneration of the hematopoietic system within the BM compartment of  $GATA-2^{+/+}$  and  $GATA-2^{+/-}$  mice was examined at days 0, 4, 8, 12, and 16 after 5-FU injection for (A) total BM cell numbers, (B) the frequency of CFU-GM in  $10^4$  BM cells, and (C) the total number of CFU-GM in the BM (two tibias plus two femurs). The regeneration of BM HSCs was examined by quantification of the Sca-1<sup>+</sup> and c-kit<sup>+</sup> cells within the lin<sup>-</sup> population by flow cytometry. (D) Percentage and (E) absolute number of LSK BM cells was examined at days 0, 4, 8, 12, and 16 after 5-FU injection.  $GATA-2^{+/+}$  samples are represented by ●, and  $GATA-2^{+/-}$  samples are represented by ○. Two complete time course experiments were performed. Each point represents an average of two to three animals with SEM. \*Significant difference between the  $GATA-2^{+/+}$  and  $GATA-2^{+/-}$ . (B) CFU-GM frequency day 12,  $P < 0.008$ . (C) CFU-GM numbers day 12,  $P < 0.002$ . (E) LSK cell number day 8 and 12,  $P < 0.04$ .

$2^{+/-}$  FL. Similarly, no difference in the percentages of HSCs as defined by the Lin<sup>-</sup>Sca-1<sup>+</sup>c-kit<sup>+</sup> (LSK) phenotype was found when  $GATA-2^{+/+}$  and  $GATA-2^{+/-}$  adult BM was analyzed (Fig. 5 C). Together, these phenotypic data support the transplantation data in showing that HSCs are quantitatively decreased in the AGM but that HSCs increase to normal numbers in the FL and adult BM.

**Cytotoxic Drug Treatment Reveals a Proliferation Defect in  $GATA-2^{+/-}$  BM HSCs.** To test if the qualitative defect in  $GATA-2^{+/-}$  HSCs is related to proliferation,  $GATA-2^{+/+}$  and  $GATA-2^{+/-}$  mice were treated with the cytotoxic drug 5-FU (28). At 0, 4, 8, 12, and 16 d after treatment, BM cells were tested in in vitro assays and analyzed by flow cytometry for evidence of hematopoietic regeneration. As shown in Fig. 6 A, both  $GATA-2^{+/+}$  and  $GATA-2^{+/-}$  mice showed similar reductions in total BM cell number at 4 d post 5-FU. Total BM cell numbers increased to starting numbers by 16 d post-5-FU. No significant differences were observed between the number of total  $GATA-2^{+/+}$  and  $GATA-2^{+/-}$  BM cells at any time point. In addition, no defect in hematopoietic differentiation was observed in  $GATA-2^{+/-}$  BM cells, as flow cytometric analysis showed the presence of all three lineages, lymphoid, myeloid, and erythroid at similar levels in  $GATA-2^{+/+}$  and  $GATA-2^{+/-}$  BM (not depicted).

To investigate whether specific immature hematopoietic progenitors were affected in 5-FU-treated  $GATA-2^{+/-}$  mice,

we performed in vitro colony assays for CFU-GM. As shown in Fig. 6, B and C, the starting frequency and number of CFU-GM were the same in  $GATA-2^{+/+}$  and  $GATA-2^{+/-}$  BM. At 4 d post 5-FU treatment, frequency and number of CFU-GM reached a similar low point in both  $GATA-2^{+/+}$  and  $GATA-2^{+/-}$  BM. However, at 12 d after 5-FU treatment, CFU-GM frequency and number in  $GATA-2^{+/+}$  BM reached higher or the same levels as the untreated BM, whereas the  $GATA-2^{+/-}$  BM CFU-GM frequency and number remained low ( $P < 0.008$  and 0.002, respectively). Only at day 16 after 5-FU treatment did  $GATA-2^{+/-}$  CFU-GM frequency and number reach the same levels as in untreated BM. Thus, the 4 d delay in the regeneration of CFU-GM in  $GATA-2^{+/-}$  BM suggests a  $GATA-2$  dose-related proliferation defect acting within these progenitors. Alternatively, a  $GATA-2$  dose-related proliferation defect acts within HSCs and only secondarily influences CFU-GM.

To test this, we analyzed the regeneration of HSCs. We performed flow cytometric analysis for LSK BM cells at 0, 4, 8, 12, and 16 d post-5-FU treatment. In both  $GATA-2^{+/+}$  and  $GATA-2^{+/-}$  BM, HSC percentages and absolute numbers were similarly reduced at 4 d post-5-FU treatment (Fig. 6, D and E). Both  $GATA-2^{+/+}$  and  $GATA-2^{+/-}$  BM HSC percentages and numbers began recovering at day 8 post-5-FU, when they surpassed the initial percentages and numbers. However,  $GATA-2^{+/-}$  BM HSC numbers remained significantly lower than in  $GATA-2^{+/+}$  BM at both day 8

and 12 post-5-FU treatment ( $p < 0.04$ ). Furthermore, whereas  $GATA-2^{+/+}$  HSC numbers peaked at day 12 post-treatment and declined thereafter,  $GATA-2^{+/-}$  HSC numbers increased slowly up to day 16 post-5-FU treatment. The finding that HSC expansion in 5-FU-treated  $GATA-2^{+/-}$  mice is delayed by at least 4 d strongly suggests a  $GATA-2$  dose-dependent proliferation defect in HSCs.

## Discussion

The data presented here show for the first time that a full dose of  $GATA-2$  is required during embryonic and adult stages for quantitatively and qualitatively normal HSC activity in vivo. Although HSCs are most likely not produced in  $GATA-2^{-/-}$  embryos, the effects of  $GATA-2$  haploinsufficiency had been largely unexplored because such  $GATA-2^{+/-}$  animals grow normally and present an overtly normal adult hematologic profile. Here we have shown that with only half the dose of  $GATA-2$ , HSC numbers are severely and specifically reduced in the AGM region, where the first induction and expansion of HSCs is occurring during midgestation. Moreover, AGM HSC quality is compromised. Thereafter, in the other tissues harboring HSCs, quantitative deficiencies in  $GATA-2^{+/-}$  HSCs appear to be compensated through normal (albeit delayed) expansion of HSCs, but qualitative deficiencies are retained through to adulthood. Therefore, given that only a few HSCs out of the whole cohort of HSCs are actively contributing to the hematopoietic system at any one time (29, 30) and that  $GATA-2^{+/-}$  cells are not defective in differentiation, no general hematologic defects would be expected in haploinsufficient adults. Only through stringent in vivo transplantations or cytotoxic stress are HSC functional defects observable. The results of these experiments strongly suggest an essential role for  $GATA-2$  in the induction and expansion of the first HSCs in the AGM and an additional, distinctive role for  $GATA-2$  in the proliferation of HSCs.

**HSC Quantitative Processes Are Altered in  $GATA-2^{+/-}$  Mice.** In in vivo transplantation experiments we show quantitatively reduced HSC activity in  $GATA-2^{+/-}$  embryos. The four- to ninefold decrease in HSC activity in E11/E12  $GATA-2^{+/-}$  AGMs compared with  $GATA-2^{+/+}$  AGMs is the consequence of fewer HSCs, as aorta sections and flow cytometric analysis show a sixfold decrease in phenotypically defined HSCs. Hence,  $GATA-2^{+/-}$  AGMs can neither expand nor maintain HSCs compared with  $GATA-2^{+/+}$  AGMs. In contrast, HSCs are expanded and maintained in E11/E12  $GATA-2^{+/-}$  YS (at a slightly decreased number), with the fold decrease in phenotypically defined HSCs in the YS corresponding closely to the fold decrease in HSC activity. Considering the fact that HSCs are first detected in the AGM region and then in the YS and FL, the reduced HSC content of the  $GATA-2^{+/-}$  YS and FL may well be a secondary effect of the reduction in the  $GATA-2^{+/-}$  AGM region.

Our in vivo analyses for hematopoietic progenitor cells in the AGM region and the YS show that CFU-S<sub>11</sub> are also

$GATA-2$  dose dependent. These data are consistent with previous in vitro studies on  $GATA-2^{-/-}$  YS and ES cells (13, 31), showing much reduced hematopoietic progenitor activity. The reduced CFU-S<sub>11</sub> activity in  $GATA-2^{+/-}$  AGMs and YSs could further be a consequence of the reduced HSC activity we detected in these tissues. However, the source of cells providing the CFU-S<sub>11</sub> activity in the embryo is not clear. Whereas in the adult, hematopoietic progenitor cells are derived from HSCs, in the preE10.5 AGM region and the YS they may be derived from hemangioblasts and/or hemogenic endothelium rather than via a HSC ancestor. Hence,  $GATA-2$  may act directly on the in vivo generation, survival, and/or expansion of the hematopoietic progenitor cells, HSCs, and/or their direct precursors in the AGM and YS.

**The YS as a Compensatory Generator of HSCs Independent of  $GATA-2$  Dose.** For over three decades, the origins of adult HSCs have been a focus of research. The view that the mammalian YS is able to provide hematopoietic cells that migrate and colonize the FL and then the BM during the neonatal/adult stages has been altered by the finding that the first fully functional adult HSCs are autonomously generated in the AGM region. Shortly thereafter, the YS contains HSCs, but due to the experimental constraints of mammalian embryos, it is difficult to definitively demonstrate whether these HSCs are AGM derived or autonomously generated in the YS. Recent data suggest that indeed YS can autonomously generate and expand HSCs (6) and putative pre-HSCs (32). Since we observe a dramatic reduction of HSCs in  $GATA-2^{+/-}$  AGMs but only a slight reduction of HSCs in  $GATA-2^{+/-}$  YSs, our transplantation data support the notion of YS HSC generation potential (albeit in a  $GATA-2^{+/-}$  embryo). However, since HSCs are still generated in the  $GATA-2^{+/-}$  AGM 1 d earlier than in the YS, it remains possible that YS HSCs are AGM derived. Interestingly, the reduced HSC activity in the  $GATA-2^{+/-}$  YS can be expanded to a magnitude comparable to that of the  $GATA-2^{+/+}$  YS in explant cultures, suggesting that at least some of the reduced activity in the  $GATA-2^{+/-}$  YS is a secondary effect of the reduction of HSCs in the AGM. Furthermore,  $GATA-2^{+/-}$  HSC numbers are compensated to normal levels in the adult, possibly due to HSC generation and expansion in the YS and the further expansion in the FL and BM. Notwithstanding, these data imply that the underlying molecular mechanisms in which the AGM generates, maintains, and expands HSCs are different from that of the YS. The AGM region is exquisitely sensitive to the level of the  $GATA-2$  dose, whereas the YS is much less sensitive. Hence, the HSC defects in  $GATA-2^{+/-}$  AGMs do not result in severe anemia in adults since  $GATA-2^{+/-}$  YS can generate and/or expand sufficient HSCs irrespective of the haploinsufficiency.

**HSC Qualitative Processes Are Altered in  $GATA-2^{+/-}$  Mice.** The results of adult BM competitive transplantation experiments clearly demonstrate a qualitative difference between the  $GATA-2^{+/+}$  and  $GATA-2^{+/-}$  HSCs. The high percentage of  $GATA-2^{+/-}$  mice engrafted with  $GATA-$

$2^{+/-}$  cells, even at low donor cell numbers, demonstrate that  $GATA-2^{+/-}$  BM HSCs have a proliferative advantage over the  $GATA-2^{+/-}$  BM HSCs. In the reciprocal transplantation in which  $GATA-2^{+/-}$  BM cells were transplanted into  $GATA-2^{+/-}$  recipients, high numbers of cells were needed to obtain a high percentage of donor-engrafted recipient mice and thus imply that: (a) the number of HSCs in  $GATA-2^{+/-}$  BM is quantitatively reduced; (b) the  $GATA-2^{+/-}$  HSCs have a lower proliferative advantage over the  $GATA-2^{+/-}$  HSCs; and/or (c) the homing efficiency is lower for  $GATA-2^{+/-}$ -derived HSCs. The fact that no significant difference in the percentage or absolute number of LSK BM cells was found between  $GATA-2^{+/-}$  and wild-type BM indicates that the decreased HSC activity is not due to a quantitative decrease in  $GATA-2^{+/-}$  BM HSCs. However, the delayed expansion of HSCs in the 5-FU recovery experiments does strongly suggest that the major  $GATA-2$  dose-dependent defect is in HSC proliferation. Although homing of HSCs is not required in this experimental scenario, we cannot exclude an additional  $GATA-2$  dose-dependent defect in homing.

*How Does GATA-2 Dose Affect the Quantitative and Qualitative Development of HSCs?* We propose here that the  $GATA-2$  dose effects we observe in the AGM act at the level of the hemogenic cells that differentiate into HSCs. Normally, a full dose of  $GATA-2$  is required for the generation, maintenance, and/or expansion of these precursor cells. In the haploinsufficient AGM region, these hemogenic cells fail to differentiate, survive, and/or divide. However, owing to the stochastic nature of gene expression, some hemogenic cells still achieve a threshold level of  $GATA-2$  protein, and therefore, the target genes (which are needed for the differentiation, survival and/or division of the precursor cells) can be activated at some low frequency in the E10.5 AGM. The outcome of  $GATA-2$  haploinsufficiency is then a small production of HSCs followed by an overall reduction in the absolute number of AGM HSCs that we can detect functionally in our transplantation assay and phenotypically in immunostained sections and flow cytometry analysis.

Recent  $GATA-2$  expression data in the AGM support the notion of a role for  $GATA-2$  in hemogenic precursors. Transgenic embryos with a GFP marker under the control of  $GATA-2$  transcriptional regulatory sequences show high levels of  $GATA-2$  expression in  $CD45^{-}$  AGM cells with hemogenic potential and a significant decrease in the percentage of  $CD45^{+}$  cells in  $GATA-2^{+/-}$  E11.5 AGMs (12). Moreover, during midgestation, at the time of the first induction of HSCs,  $GATA-2$  is expressed in the endothelial cells lining the dorsal aorta and some underlying mesenchymal cells. Hence, high  $GATA-2$  expression in hemogenic cells of the AGM suggests that  $GATA-2$  is acting on the cells just immediately preceding the induction of HSCs.

Since  $GATA-2$  is a transcription factor, its target genes within hemogenic AGM cells are of particular interest. Several markers of AGM HSCs and aortic hemogenic cells have been recently described: the  $Ly-6A$  (Sca-1) cell surface

glycoprotein (23, 33) and  $Runx-1$  transcription factor (27). These molecules are overlapping with  $GATA-2$  in their expression patterns in hemogenic cells of the dorsal aorta. Targeted mutation of these genes results in qualitative and/or quantitative defects in HSCs. Whereas  $Ly-6A^{-/-}$  embryos thrive into adulthood with no or little effect on HSC generation in the embryo, functional analyses of HSCs derived from  $Ly-6A^{-/-}$  mutant BM show defects in their self-renewal ability (34), similar to our findings in  $GATA-2^{+/-}$  BM. In contrast,  $runx-1^{-/-}$  embryos are completely devoid of HSCs and exhibit FL anemia leading to lethality at E12 (35–37). Moreover,  $runx-1$  haploinsufficiency leads to a premature extinction of AGM HSCs (20). Hence, the  $Ly-6A$  and  $runx-1$  genes could be targets of  $GATA-2$  or, alternatively, contribute to the activation of the same pathways for HSC self-renewal and/or HSC generation. At present, although many  $GATA$  consensus-binding sites appear in the sequences surrounding these genes, there is no *in vivo* data showing that any of these sites are functional.

Nonetheless, two bona fide target genes of  $GATA-2$  have been proposed. These are  $SCL/tal-1$ , an essential early hematopoietic transcription factor and  $E4bp4$ , a transcription factor implicated in cell survival. *In vivo* studies show that  $GATA-2$  forms a multiprotein complex with  $Fli-1$  and  $Elf-1$  that binds the  $SCL$  enhancer and activates the expression in HSCs, endothelial cells, and their bipotent progenitor, the hemangioblast (38). However, *in vivo* mutation analysis on the HSC-specific  $GATA$  sites within the  $SCL$  locus affects  $SCL$  expression not only in the AGM but also in YS and FL (38). Therefore, it is unlikely that the selective defect in the  $GATA-2^{+/-}$  AGM HSCs can be attributed to defective  $SCL$  expression. Chromatin immunoprecipitation studies on BaF3 cell line stimulated with IL-3 show that  $GATA-2$  binds to a sequence downstream of the transcriptional start site of  $E4bp4$  and is necessary for transcriptional activation of this gene (39). Considering that IL-3 is a survival factor for HSCs, it is plausible that  $GATA-2$  is involved in the activation of this pathway.

In conclusion,  $GATA-2$  dosage is important in regulation of HSC production and expansion. Haploinsufficiency of  $GATA-2$  results in quantitative decreases in HSCs in the AGM and qualitative defects in HSCs in both the embryonic-derived and adult BM HSCs. The pivotal importance of  $GATA-2$  in these processes within HSCs now awaits the identification of the relevant target genes and the functional cascades that  $GATA-2$  activates, most likely in concert with other factors in multiprotein complexes.

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